Effects of Borate and Other Ions on the Alkaline Phosphatase of Bovine Milk and Intestinal Mucosa

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Introduction

The inhibitory effect of borate on alkaline phosphatase has frequently been noted (1–6), but no quantitative studies have been reported. Such a study appeared to be desirable because borate reacts with riboflavin (7), pyridoxine (8), muscle adenylic acid (9), and polysaccharides (5, 10, 11), and inhibition of alkaline phosphatase might be due to interaction with some such component of the enzyme.

Preliminary to the study of the effect of the borate, investigations were made to find a suitable buffer in which to perform the enzymatic reaction. Ethanolamine and its hydrochloride were chosen as being most satisfactory. The relative inhibition of the phosphatase by certain anions other than borate was also determined.

Methods

Preparation of the Alkaline Phosphatase of Milk

Unpasteurized cow's milk was treated with rennet, and the coagulum of casein was removed by filtering through cheesecloth. Solid ammonium sulfate was added to the filtrate until the concentration was 2.3 *M*. The precipitate was redissolved in water to give a protein concentration of about 3%, and digested with trypsin until the precipitation with trichloroacetic acid had decreased about two-thirds. This digest was mixed with an equal volume of 1.5 *M* Na_2SO_4 and 0.2 volume of toluene, shaken for several hours, and filtered. An equal volume of 1.5 *M* Na_2SO_4 was added to the filtrate, and the precipitate was discarded. Twenty g. of ammonium sulfate was added to each 100 ml. of solution. The precipitate was dissolved, dialyzed to reduce the salt concentration, and kept at 7°C., pH 7.5 to 8.5, with chloroform present. The solution

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contained 0.25% protein (Biuret), and when assayed in a dilution of 1:25, gave a reading of 16.3. The activity decreased about 10% in 2 weeks.

This method resulted in a purification of about 60-fold and removed a lipide fraction which always accompanied the enzyme in other methods of purification tried. For maximum stability during the purification, the pH was kept in the range 7.5 to 8.5.

Preparation of the Alkaline Phosphatase of Intestinal Mucosa

Preparation of this material from the intestinal mucosa of the calf has been described (12). A stock solution, prepared by dissolving 10 mg. in 99 ml. of water and 1.0 ml. of 0.1 *M* sodium veronal (final pH about 8.6) and adding 0.5 ml. of chloroform, had not changed in activity in 6 months at 7°. One ml. of a 1:25 dilution gave a reading of 29.0 in the assay procedure.

Assay of Alkaline Phosphatase

The substrate used was sodium phenylphosphate in a concentration of 0.00075 *M*. The phenol released by the enzyme was determined with the reagent of Folin and Ciocalteu (13). In the assay, 0.9 ml. of 0.01 *M* substrate is diluted with 8.1 ml. of 0.1 *M* buffer, and 0.2 ml. of 0.15 *M* $MgCl_2$ and 1.8 ml. of water is added. The temperature of this mixture is raised to 37°, and 1.0 ml. of enzyme is added. After the enzyme has acted for 5 min., 4.0 ml. of the Folin reagent is added, stopping the reaction. The phenol color is developed by adding 6.4 ml. of 1.9 *M* Na_2CO_3 and holding at 37° for 30 min. The blue colors are read on the logarithmic scale in a photometer with a No. 650 filter. With the milk enzyme, an inactive-enzyme blank is used; with the mucosa enzyme, a reagent blank gives the total extraneous color. The results are expressed as photometer readings or as moles of phenol liberated. When the 0.1 *M* ethanolamine buffer is employed, a reading of 14.3 is equivalent to 1.0×10^{-7} moles of phenol; in 0.05 *M* ethanolamine buffer, the reading is 13.0; with all the other buffers and anions studied, a reading of 12.0 is obtained with the same amount of phenol. The data in almost every case represent duplicate analyses. The hydrolysis of the substrate was less than 5% except in the few experiments with extremely dilute substrate shown in Fig. 4.

Results

The assay values given under *Methods* were obtained at pH 9.67 with 0.1 *M* (0.068 *M* final concentration) ethanolamine–HCl buffer (pK of ethanolamine is 9.5). Sodium veronal, used in initial studies, gave variable final pH values. Veronal and several other buffers used for the assay of alkaline phosphatase were investigated in respect to variation in enzyme activity with change of pH. Figure 1 gives the results. One ml. of the enzyme diluted 1:25 was used, except in the experiment with mucosa enzyme in borate, in which the dilution was 5:25. All data are expressed on a 1:25 basis since experiments with several concentrations

of the enzyme showed that the activity was proportional to the concentration. The data obtained with the mucosa and the milk enzymes are similar, but the optima for the milk enzyme lie at slightly higher pH values, and the order of activity in the veronal and ethanolamine buffers is reversed. Consideration of the effect on the enzyme action, and pH optimum in relation to maximum buffering action, led to the main use of the ethanolamine buffer for subsequent studies.

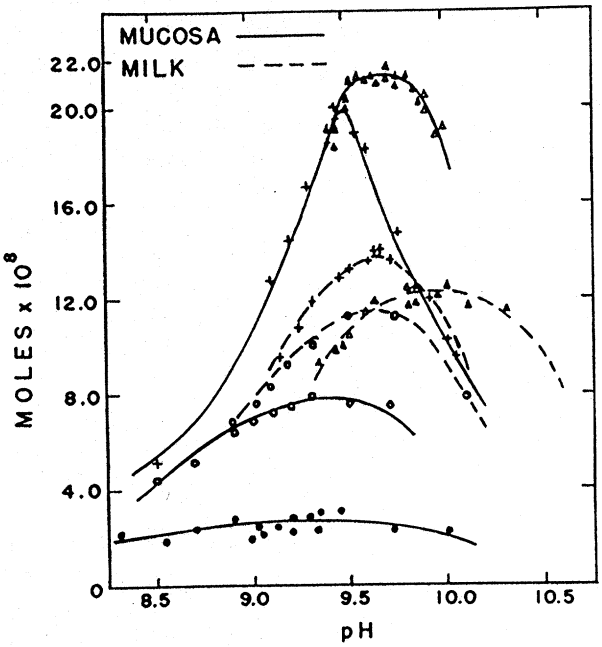

FIG. 1. Activity, in moles of phenol liberated, of the alkaline phosphatase of bovine milk and intestinal mucosa in relation to pH in various buffers. △, 0.068 *M* ethanolamine–HCl; +, 0.068 *M* sodium veronal–HCl; ○, 0.017 *M* sodium tetraborate–HCl; ●, 0.068 *M* sodium tetraborate–HCl.

The effect of borate (this term in every case refers to sodium tetraborate) on the mucosa enzyme in ethanolamine (0.068 *M*, pH 9.67) and carbonate (0.068 *M*, pH 9.37) buffers was investigated. The borate replaced the water in the assay method; final pH values were checked. Figure 2 shows the data obtained. Carbonate was inhibitory to the mucosa phosphatase (Table I), but this does not show in the figure, for

the same base line was chosen as for the ethanolamine buffer. From the data, it can be seen that 0.011 M tetraborate caused 50% inhibition (ordinate = 6.9) of the mucosa enzyme in carbonate; extrapolation of the data shows that 0.019 M would be required to give 50% inhibition in ethanolamine. This inhibitory concentration of borate is about the same as that calculated from the activity in borate buffers (Fig. 1) compared with the activity in veronal and ethanolamine. The nonlinear inhibition curve for borate in carbonate, which indicates that in carbonate buffer borate is less inhibitory in dilute solution than in more concentrated solutions, suggests that the borate and carbonate might be competing for the same sites on the enzyme. This was another reason for the choice of the ethanolamine buffer.

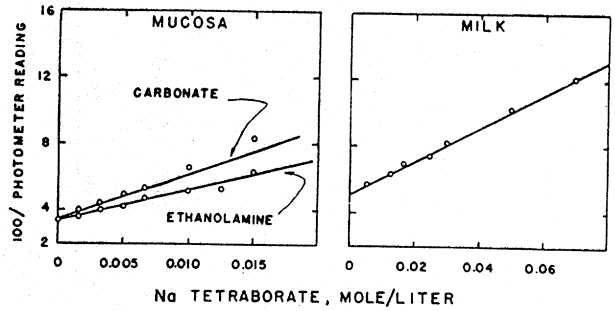

FIG. 2. Effects of borate on mucosa phosphatase in carbonate and ethanolamine buffers, and the effects of borate buffers on milk phosphatase.

Study of the effect of borate on the milk phosphatase showed that this enzyme was much less inhibited than the mucosa enzyme. Since adequate buffering was provided by the concentrations of borate used, the ethanolamine was omitted. Inhibition by borate was of the same magnitude, with or without ethanolamine. Figure 2 shows these data. The concentration of tetraborate giving 50% inhibition (ordinate = 10.0) was 0.050 M.

The effects of 0.005 and 0.01 M tetraborate on the mucosa phosphatase in 0.071 M ethanolamine at pH 9.6 with several concentrations of substrate ($1.53–11.3 \times 10^{-4}$ M) were also studied. In Fig. 3, the reciprocals of the reaction velocities ($1/V$; V = moles/5 min.) are plotted against the reciprocals of the concentrations of substrate ($1/C$). Also presented are the data for the reaction in 0.035 and 0.071 M ethanol-

amine with no borate present. On the basis of the analysis of enzyme inhibitions given by Lineweaver and Burk (14), and recently by Harmon and Niemann (15), the intercept of the curve on the abscissa equals $1/V_{max.}$, where $V_{max.}$ is the maximum activity with excess of substrate. The slope of the curve equals $K_s/V_{max.}$ where K_s is the constant for the enzyme–substrate dissociation, and numerically is the concentration of substrate giving one-half the maximum activity. In competitive inhibition, the slope increases and with it the apparent K_s, with no change in the intercept. It can be concluded that the borate

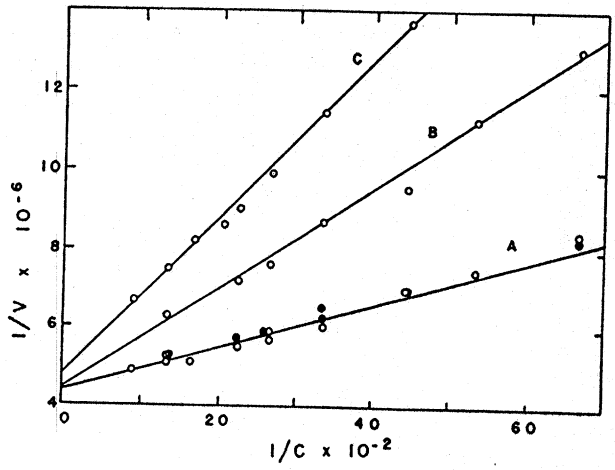

FIG. 3. Effect of borate on mucosa phosphatase (1:25) in relation to concentration of substrate. Velocity (V) is expressed in moles/5 min.; concentration of substrate (C) is in moles/l. A, no borate: ○, 0.035 M ethanolamine; ●, 0.071 M ethanolamine; B, 0.005 M tetraborate; C, 0.01 M tetraborate.

inhibition is of this type, inhibition being greatest with low concentrations of substrate. From the data with no borate, the value of $V_{max.}$ is 2.27×10^{-7} moles, and the enzyme–substrate dissociation constant K_s is 0.00013 mole/l. There is a slight shift of the $1/V$ intercept in the presence of borate, the respective $V_{max.}$ for 0.005 and 0.01 M tetraborate becoming 2.22×10^{-7} and 2.08×10^{-7} moles; the apparent K_s values are 0.00028 and 0.00042 mole/l. The linear extrapolation of these K_s values to zero borate concentration gives a value of 0.00015 mole, in good agreement with that found in the borate-free solutions.

The shift of the intercept on the $1/V$ axis is evidence for a slight noncompetitive inhibition. Jacobsen (16) has reported that the inhibition of alkaline phosphatase by phosphate, although largely competitive, is in part noncompetitive.

Since the results for both concentrations of ethanolamine fall on the same straight line, there is no inhibition of either the competitive or noncompetitive type when the concentration of this buffer is increased.

A similar analysis of the milk phosphatase was made in borate buffers at pH 9.6 (Fig. 4). The phosphatase was diluted 2:25 for these experiments; the same K_s values were obtained with a 1:25 dilution of the phosphatase. The following data were obtained for 0.035, 0.021, and 0.014 M borate. $V_{max.}$ values: 2.56, 2.90, and 3.13 $\times 10^{-7}$ moles,

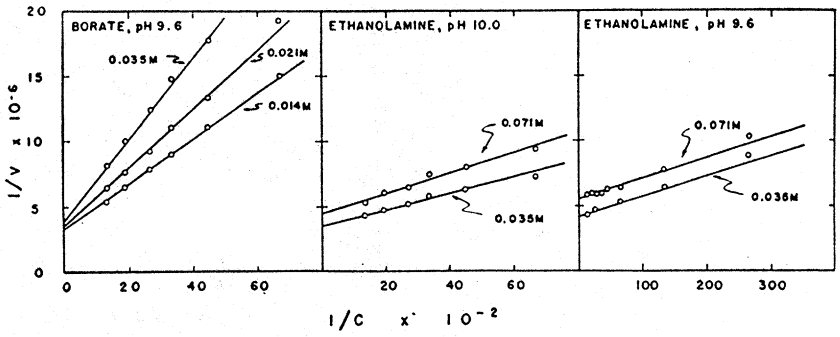

FIG. 4. Effects of borate and ethanolamine on milk phosphatase (2:25) in relation to concentration of substrate. Velocities (V) are expressed in moles/5 min.; concentration of substrate (C) in mole/l.

respectively; apparent K_s values: 0.00082, 0.00064, and 0.00053 mole/l, respectively. The value of K_s from an extrapolation of the apparent values to zero borate concentration is 0.00035 mole/l.

The constant K_i for the dissociation of the enzyme–inhibitor complex was calculated from the borate inhibition substrate concentration data by the procedure described by Harmon and Niemann (15). For the mucosa phosphatase, K_i is 0.0036 mole/l; for the milk phosphatase, 0.012 mole/l.

The milk enzyme was also studied at several concentrations of substrate in 0.071 and 0.035 M ethanolamine at pH 9.6 and 10.0, respectively. These data are included in Fig. 4. It is apparent that the milk enzyme is inhibited by ethanolamine and that it is a noncompetitive

inhibition, that is, the K_s values are essentially the same for the two concentrations of ethanolamine. With the 0.00075 M substrate at both pH values, the inhibition produced by doubling the ethanolamine concentration is 20%. Calculations like those employed with the data in Fig. 2 indicate that the enzyme would be inhibited 50% at concentrations of 0.073 and 0.105 M ethanolamine at pH 9.6 and 10.0, respectively.

In noncompetitive inhibition, $1/V\ (1 + i/K_i) = 1/V_i$, where V_i is the maximum velocity in the presence of inhibitor, and the inhibitor–

TABLE I

Inhibition of Alkaline Phosphatase by Certain Ions

Ions	Concentrations tested	Concentrations giving 50% inhibition[a]	
		Mucosa enzyme	Milk enzyme
	mole/l.	*mole/l.*	*mole/l.*
Tetraborate	0.0015–0.011 (mucosa) .0125– .100 (milk)	0.019	0.052
Phosphate	.004 and .008	.0013	.0108
Pyrophosphate	.004 and .008	.0013	.0120
Carbonate	.04 and .08	.21	—
Arsenate	.0004 and .0008	.00013	.00071
Cacodylate	.02 and .04	.095	—
Ethanolamine	.035 and .071	No inhibition	.09

[a] Determined from graphs of the type used in Fig. 2.

enzyme constant K_i is $(1/V)\ (i)/(1/V_i - 1/V)$. From a plot of $1/V_i$, the intercept in the presence of inhibitor, the value of the intercept when no inhibitor is present, $1/V$, can be found by extrapolation. By this means, K_i values were calculated for inhibition by ethanolamine of 0.071 mole/l. at pH 9.6; 0.085 at pH 10.0.

At pH 10.0, the optimum pH for the milk enzyme in ethanolamine, the K_s is 0.00020 mole/l., about the same as the K_s in borate (extrapolated to borate = 0) at pH 9.6, the optimum in borate. There is a great dependence of the K_s on pH, for at pH 9.6 in ethanolamine it is sixfold smaller than at pH 10, that is, 0.000034 mole/l. Milk and the mucosa enzymes have about the same K_s values at their pH optima (pH 10.0

and 9.6, respectively) in ethanolamine, but when compared at the same pH (9.6) they differ fourfold.

The effects on alkaline phosphatase of the anions phosphate, pyrophosphate, carbonate, and arsenate have frequently been studied. Table I gives the inhibition of these anions on the phosphatases used in the present studies for comparison with the inhibition by tetraborate.

Discussion

Both milk and mucosa phosphatases are inhibited competitively by sodium tetraborate. Since both phosphate (16) and carbonate (17) also inhibit alkaline phosphatase competitively, it is likely that the borate inhibition is of a similar anionic type rather than due to its ability to react with the polyhydroxy compounds mentioned in the introduction. It is of interest that borate has been found inhibitory to the enzymes urease (18), arginase (19), and pepsin (20). However, none of these contains riboflavin, pyridoxine, or adenylic acid, and it is probable that this inhibition is of the competitive anionic type, since urease, for example, is inhibited competitively by phosphate (15). It may be significant that with arginase, which is activated by manganese, the inhibition by borate is greater than that by phosphate, which is the reverse of that obtained with the magnesium-activated alkaline phosphatase. Tetraborate (0.033 M) does not inhibit the riboflavin-containing xanthine oxidase from milk (21), but it would be of interest to see borate tested on other enzymes containing riboflavin, as well as pyridoxine and adenylic acid.

Published enzyme–substrate constants (K_s) are difficult to compare because of the influence of pH, buffers, and the kind of substrate. However, the values reported here are of the same magnitude as those reported by others for the hydrolysis of phenyl phosphate. Folley and Kay (22) obtained a value of 0.0006 with the phosphatase of mammary tissue in glycine buffer at pH 10.0. They observed the decrease in K_s with decrease in pH. The great effect of pH on K_s was observed by Jacobsen (16) with kidney phosphatase. Schmidt and Thannhauser (23) using intestinal phosphatase at a pH of 9.3 in veronal buffer obtained a value of 0.000072 for K_s. This value is lower than that obtained in the present studies, but this is to be expected because of the lower pH.

Inhibition of milk phosphatase by ethanolamine is of the noncom-

petitive type, which is the same type shown by the ammonium ion (17). Inhibition of alkaline phosphatase by glycine is also predominantly of the noncompetitive type (24).

The effects of different buffers on the pH optima of kidney (rat) alkaline phosphatase when acting on glycerophosphate have recently been reported (6). The data parallel those of the present study, in that glycine and NH_4OH buffers, which may be compared with ethanolamine, give the highest pH optima, next come veronal, and then borate. The results are comparable too in the broad pH optima for NH_4OH, ethanolamine, and borate and the sharp pH optimum for veronal. The effects of the buffers on the activity are also of about the same general magnitude.

Ethanolamine is an excellent buffer for alkaline phosphatase assays for the reasons mentioned earlier. Further, phosphatase in it has a broad pH optimum, which is desirable. Experiments at pH 9.6 are at the optimal pH for the mucosa phosphatase and only slightly below the optimum for the milk phosphatase; buffering is maximal, and in the case of milk enzyme, because of the low K_s value, low concentrations of substrate are adequate.

A number of means have been tried to reduce the borate inhibition of mucosa phosphatase to that shown by the milk phosphatase. Heat-inactivated milk enzyme, acid-inactivated mucosa enzyme, alanine, and magnesium were added or varied without changing the inhibition. The phosphatase in whey showed the same inhibition as did the purified phosphatase. It is probable that the difference in behavior of the two enzymes is not due to the presence of a second admixed component. However, it is possible that a second component may have reacted with the one enzyme to give the other. Both enzymes give broad pH optima with borate; both give sharp optima with veronal and both have about the same K_s values at their optimal pH in ethanolamine.

Some of the data, however, suggest that milk and mucosa phosphatases are different enzymes. The milk enzyme has a higher pH optimum than the mucosa enzyme; at pH 9.6 it has a much lower K_s value, and it is less inhibited by anions and more inhibited by cations (ethanolamine). Various means have been tried for distinguishing several alkaline phosphatases. Further investigation with phosphatases from different tissues might show that the comparative effects of anions and cations would provide a means for distinguishing two types. Data

recently reported by Bodansky (25) conform with this suggestion. The cationic amino acids histidine and lysine were about three times more inhibitory to bone and kidney phosphatases than they were to intestinal mucosa phosphatase, whereas the anionic glutamic acid was about three times more inhibitory to the mucosa phosphatase than to the other phosphatases. On this basis, the milk phosphatase would be classified with kidney and bone phosphatases. Folley and Kay (22) have noted the close resemblance of kidney and mammary phosphatases on other points.

Summary

Alkaline phosphatases from bovine milk and intestinal mucosa are inhibited competitively by borate, probably by an ionic effect. Milk phosphatase is inhibited noncompetitively by ethanolamine. Enzyme–substrate dissociation constants (K_s) and enzyme–inhibitor constants (K_i) have been calculated.

Differences in pH optima, the relative inhibition by borate and ethanolamine, and K_s values indicate that the phosphatase activity of milk and mucosa represent two different enzymes. It is suggested that the relative inhibition by anions and cations might distinguish intestinal phosphatase from milk, kidney, and bone phosphatases.

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